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Discerning the Role of *Bacteroides fragilis* in Celiac Disease Pathogenesis

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Celiac disease (CD) is associated with intestinal dysbiosis, which can theoretically lead to dysfunctions in host-microbe interactions and contribute to the disease. In the present study, possible differences in *Bacteroides* spp. and their pathogenic features between CD patients and controls were investigated. *Bacteroides* clones ($n = 274$) were isolated, identified, and screened for the presence of the virulence genes (*bft* and *mpII*) coding for metalloproteases. The proteolytic activity of selected *Bacteroides fragilis* strains was evaluated by zymography and, after gastrointestinal digestion of gliadin, by high-pressure liquid chromatography/electrospray ionization/tandem mass spectrometry. The effects of *B. fragilis* strains on Caco-2 cell culture permeability and inflammatory response to digested gliadin were determined. *B. fragilis* was more frequently identified in CD patients than in healthy controls, in contrast to *Bacteroides ovatus*. *B. fragilis* clones carrying virulence genes coding for metalloproteases were more abundant in CD patients than in controls. *B. fragilis* strains, representing the isolated clones and carrying metalloprotease genes, showed gelatinase activity and exerted the strongest adverse effects on the integrity of the Caco-2 cell monolayer. All *B. fragilis* strains also showed gliadin-hydrolyzing activity, and some of them generated immunogenic peptides that preserved or increased inflammatory cytokine production (tumor necrosis factor alpha) and showed increased ability to permeate through Caco-2 cell cultures. These findings suggest that increased abundance of *B. fragilis* strains with metalloprotease activities could play a role in CD pathogenesis, although further *in vivo* studies are required to support this hypothesis.

Celiac disease (CD) is the most common chronic intestinal inflammatory disorder triggered by ingestion of dietary gluten. This disease is considered as both a food hypersensitivity and an autoimmune disorder that involves genetic and environmental factors (40). The human leukocyte antigen (HLA) class II genes encoding for DQ2 and DQ8 heterodimers are the main hereditary factors predisposing to CD and are present in most CD patients (95%). Nevertheless, while 30 to 35% of the general population are carriers of these genes, only 2 to 5% actually develop CD, indicating that other factors contribute to precipitating the disease (33). The intake of gluten proteins is the critical environmental element responsible for the signs and symptoms of the disease and, in fact, typical cases manifest in early childhood after introduction of gluten into the diet. However, the disease is also being increasingly diagnosed in adulthood (5), suggesting that early exposure to gluten is not the only environmental trigger.

Gluten proteins and their toxic components (gliadins) are partially resistant to proteolytic degradation and can accumulate and interact with the small intestinal mucosa (14). Enzyme deficiency in the small intestinal mucosa of CD patients does not seem to be causally related to the disease (3). However, in CD patients, some peptides, such as the 33-mer of α-gliadin and others containing its main structural epitopes (PFPQPQLPY and PQPQLPYPQ), preferentially drive an adaptive immune response by binding to HLA-DQ2/DQ8 molecules of antigen-presenting cells and activating T-helper 1 (Th1) and Th17 inflammatory responses within the mucosa, with the resulting production of inflammatory cytokines (e.g., gamma interferon [IFN-γ] and interleukin-21 [IL-21]) leading to severe inflammation (24). Other gliadin peptides activate an innate immune response characterized by increased production of IL-15 by epithelial and antigen-presenting cells, which activate the effector function and cytotoxic activity of intraepithelial lymphocytes (15). Gliadin peptides also induce upregulation of the zonulin innate immunity pathway, which leads to increased intestinal permeability and enables paracellular translocation of gliadin and its subsequent interaction with antigen-presenting cells within the intestinal submucosa (11).

In recent years, alterations in the composition of the intestinal microbiota have been associated with CD. The bacterial numbers of the *Bacteroides-Prevotella* group or the *Bacteroides fragilis* group in CD patients have been demonstrated to be increased compared to those in healthy controls (8, 26). *Bacteroides* spp. are generally considered commensals or symbionts inhabiting the human gastrointestinal tract, representing ca. 25% of the total bacterial cells. Nonetheless, members of the normal microbiota can also potentially cause disease in cases of failure of the host defenses and major dysbiosis and can then be considered "pathobionts" (35). In spite of the abundance of *Bacteroides* spp. in the gut microbiota, their ecological distribution, composition, and impact on health remain unclear (46). Species such as *B. fragilis* and *Bacteroides vulgatus* seem to be implicated in the disruption of the integrity of the intestinal epithelial barrier, thereby contributing to the development of inflammation in experimental animal models (38, 42) and, possibly, in patients with inflammatory bowel disease (IBD) (10). The potential pathogenicity of *Bacteroides* spp. is related to the expression of a variety of virulence factors, including proteolytic and other hydrolytic enzymes (4). Enterotoxigenic *B. fragilis* (ETBF) strains produce an enterotoxin, termed *B. fragilis* toxin (BFT), which is a 20-kDa zinc-dependent metalloprotease that has been associated with diarrhea in humans and young animals (37).

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TABLE 1 Primers for PCR and sequencing used in this study

Primer	Fragment name	Fragment length (bp)	Primer sequence (5′–3′)	Reference
M13	M13		TTATGAAACGACGGCCAGT	21
Bfra531F	Bfra	289	ATACGGAGGATCCGAGCGTTA	20
Bfra766R			CTGTTTGATACCCACACT	
GBF-201			GAACCTAAAACGGTATATGT	22
GBF-312	*bft-1*	190	CCTCTTTGGCGTCGC	
GBF-322	*bft-2*	175	CGCTCGGGCAACTAT	
GBF-334	*bft-3*	287	TGTCCCAAGTTCCCCAG	
LO1	*mpII*	350	CCACCGTGCCAATGTCAGATA	23
RO1			CTGAAGAACGAGGCGGTATC	

The *B. fragilis* toxin gene (*bft*) is located in a pathogenicity island, present exclusively in ETBF strains and is associated with another gene (*mpII*) that encodes a second metalloprotease (12).

Here, we hypothesized that changes in the composition of *Bacteroides* spp. and associated virulence features can turn these commensal bacteria into pathogenic inhabitants of the human intestinal tract that, acting in consortium with gluten peptides, can contribute to CD. To address this question, we determined differences in the diversity of *Bacteroides* spp. isolated from the feces of patients with active and nonactive CD in comparison with healthy controls and evaluated their virulence features and potential participation in the generation of gliadin peptides with immunotoxic effects on intestinal epithelial cells.

MATERIALS AND METHODS

Subjects and sampling. Three groups of children were included in the present study: (i) patients with active CD ($n = 20$; mean age, 3.9 years; range, 1.0 to 8.8 years), who were on a normal gluten-containing diet, showing clinical symptoms of the disease, positive CD serology markers (anti-gliadin antibodies and anti-transglutaminase antibodies), and signs of severe enteropathy, classified as type 3 according to the Marsh classification of CD by duodenal biopsy examination; (ii) patients with nonactive CD ($n = 18$; mean age, 6.2 years; range, 3.3 to 12.2 years), who were on a gluten-free diet for at least 2 years, showed negative celiac serology markers and normal mucosa or infiltrative lesions classified as type 0-1 according to Marsh classification, and absence of disease symptoms; and (iii) healthy control children ($n = 20$; mean age, 5.7 years; range, 2.5 to 10.8 years). None of the children included in the study were treated with antibiotics for at least 1 month before the sampling time. The study was conducted in accordance with the ethical standards of the responsible institutional committees on human experimentation and in accordance with the Helsinki Declaration of 1975 as revised in 1983. Children were enrolled in the study after written informed consent was obtained from their parents.

Isolation of *Bacteroides* spp. from child feces. Fecal samples were collected in sterile containers, kept under anaerobic conditions (AnaeroGen; Oxoid, Hampshire, United Kingdom), stored at 4°C, and analyzed in less than 12 h to avoid alterations in viability of *Bacteroides* spp. Samples (2 g [wet weight]) were diluted (1:10 [wt/vol]) in phosphate-buffered saline (PBS; 130 mM sodium chloride, 10 mM sodium phosphate [pH 7.2]) and homogenized in a Lab Blender 400 Stomacher (Seward Medical, London, United Kingdom). Serial dilutions were prepared in PBS, and aliquots were plated on Schaedler agar (Scharlau, Barcelona, Spain) supplemented with kanamycin (100 mg/liter), vancomycin (7.5 mg/liter), and vitamin K (0.5 mg/liter) and then incubated under anaerobic conditions at 37°C for 48 h. In order to analyze the dominant clones in each subject (21), five presumably different individual colonies were isolated from the highest dilution plate from each subject, and their cellular morphology and Gram-staining characteristics were examined.

Strain typing and species identification. The isolated clones were identified at the species level by partial 16S rRNA gene sequencing using the primer pair Bfra531-f and Bfra766-r (45). The PCR products obtained were purified using GFX PCR DNA and a gel band DNA purification kit (GE Healthcare, Buckinghamshire, United Kingdom) for DNA sequencing. DNA sequencing was carried out by an ABI Prism 3130XL genetic analyzer (Applied Biosystems, California). The closest relatives of the partial 16S rRNA gene sequences were sought in GenBank using the basic local alignment search tool (BLAST) algorithm, and sequences with >97% similarity were considered to belong to the same species. RAPD [random(ly) amplified polymorphic DNA]-PCR was performed to differentiate the isolated clones at the strain level by colony PCR and using the M13 primer as previously described (Table 1) (7).

Pathogenicity markers and proteolytic activity of *B. fragilis* strains. The presence of *bft* and *mpII* genes was screened in all of the isolated *B. fragilis* clones by PCR (Table 1). One forward primer (GBF-201) and three reverse primers (GBF-312 for *bft-1*, GBF-322 for *bft-2*, and GBF-334 for *bft-3*) were used in the same amplification reaction for the detection of the three isoforms of the *bft* gene by multiplex PCR (17). *B. fragilis* clones were also screened for the presence of the *mpII* gene by PCR (23). PCR products were separated in a 2% agarose gel by electrophoresis and visualized by ethidium bromide staining.

B. fragilis clones were analyzed by RAPD-PCR, and nine different strains were identified (A to I). The proteolytic activity of one representative of each *B. fragilis* strains was determined in gelatin and gliadin zymograms. For this, strains were grown in brain heart infusion broth (Scharlau, Barcelona, Spain) supplemented with 0.05% (wt/vol) cysteine (Sigma, St. Louis, MO) under anaerobic conditions for 24 h. Bacterial cells were collected by centrifugation (6,000 × *g* for 15 min), washed, and resuspended in PBS at a final concentration of 10^8 CFU/ml. Cell suspensions were separated in a discontinuous SDS-PAGE system that consisted of (i) a running gel containing 15% acrylamide (pH 8.8) and either 0.5% gelatin or gliadin and (ii) a stacking gel containing 4% acrylamide (pH 6.8). Gels were run at a constant voltage (120 V) in a MiniProtean 3 cell system (Bio-Rad, Richmond, CA). Gels were washed in 2.5% Triton X-100 at room temperature for 1 h and then incubated in reaction buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$ [pH 7.4]) at 37°C overnight. Hydrolysis bands were visualized as a clear zone after Coomassie brilliant blue R-250 staining (Bio-Rad, Richmond, CA).

***In vitro* digestion of gliadins.** Gliadins were subjected to a simulated human gastrointestinal digestion as previously described (19). Aliquots (150 mg) of a commercially available extract of gliadin (Sigma) were dissolved in 3 ml of a saline solution (140 mM NaCl, 5 mM KCl [pH 3]) at 60°C for 30 min, with gentle agitation. Gastric digestion with pepsin (800 to 2,500 U/mg in 0.1 M HCl [pH 3] for 1 h), and intestinal digestion with pancreatin (4× USP specification) and bile (Sigma) in 0.1 $NaHCO_3$ at pH 7 for 2 h were conducted at 37°C with agitation. After the gastric digestion, the intestinal digestion was carried out in the upper part of a two-chamber system in six-well plates separated by a 15,000-molecular-weight cutoff dialysis membrane (Spectrum Medical, Gardena, CA). Aliquots of the

gastric digested samples were loaded into the upper chambers in the presence or absence of *B. fragilis* cell suspensions (10^8 CFU/ml) and incubated for 4 h. Then, saline solution from the basal chamber was recovered for further analysis. The total protein concentrations in both dialysates and retentates were quantified using a Lowry-based commercial kit (Sigma). The stability of bacteria during digestion was confirmed by plate counting under optimal conditions, which remained at 10^8 CFU/ml.

Reversed-phase HPLC and MS/MS analysis. Gliadin-derived peptides were analyzed after simulated gastrointestinal digestion, as described elsewhere (20). The separation was conducted in a BioBasic C_{18} column (5 μm; 4.6 by 250 mm; Thermo, Waltham, MA) using an Agilent high-pressure liquid chromatography (HPLC) system connected in-line to an Esquire-LC electrospray system equipped with a quadrupole ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). The elution phases consisted of 15% (vol/vol) acetonitrile (ACN)–0.1% (vol/vol) trifluoroacetic acid (TFA) (solvent A) and 80% (vol/vol) ACN–0.1% (vol/vol) TFA (solvent B). Aliquots (100 μl) of the dialysates were injected in each analysis. The gradient program started with 95% solvent A and 5% solvent B, and changed linearly to reach 10% solvent A and 90% solvent B within 30 min. UV absorbance was recorded at 214 nm. BioTools version 2.1 (Bruker Daltonics) software was used to process the tandem mass spectrometry (MS/MS) data and to identify peptide sequences by comparison to available gliadin sequences (accession numbers: α/β, AAZ94420; γ, AAQ63856; and ω, AAT74547). Three independent dialysates were analyzed in each case.

Caco-2 cell culture conditions. The human colon carcinoma Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD) at passage 14 and used in experiments at passages 19 to 23. Caco-2 cells were grown in Dulbecco modified Eagle medium (DMEM; AQ Media; Sigma), containing 4.5 g of glucose (Sigma/liter), 25 mM HEPES buffer (Sigma), 0.1% (vol/vol) antibiotic mixture (penicillin, streptomycin, and gentamicin; Sigma), and 10% (vol/vol) fetal bovine serum (Sigma). Cells were grown and maintained at 37°C in 5% CO_2 and 95% air, and the culture medium was changed every 2 days (19). Cells at 70% confluence were detached from the flasks by using a trypsin solution (2.5 g/liter; Sigma) and resuspended in DMEM.

Evaluation of intestinal Caco-2 cell monolayer integrity. Caco-2 cells were seeded at a density of 50,000 cells/cm^2 onto polyethylene terephthalate membrane inserts (0.4-μm pore size; Millipore, Billerica, MA) and placed in six-well plates (Costar). In this bicameral system, 1.5 ml of treatment medium was loaded into the apical compartment, and 2 ml of saline solution was loaded into the basal compartment. Cell cultures were used at 7 days after seeding.

To determine the influence of selected *Bacteroides* strains on the integrity of the intestinal cell monolayer, bacterial cell suspensions (10^8 CFU/ml) of *B. fragilis* strains A, B, C, and I grown for 20 h were prepared in DMEM (without antibiotics) and loaded into the upper chamber alone and together with the dialysates of gliadin digested in the presence of these strains. After incubation (4 h), the basal medium was recovered and mixed with 100 μl of 1 M NaOH, and the diffusion of phenol red was determined by measuring the absorbance at 558 nm.

To determine the translocation of gliadin-derived peptides, Caco-2 cells were exposed basolaterally to tumor necrosis factor alpha (TNF-α) (10 ng/ml) for 24 h to simulate inflammatory conditions (31). *In vitro* digestions of gliadins in the presence of cell suspensions of *B. fragilis* A, B, C, or I strains were loaded into the upper chamber of the *in vitro* system. After 4 h of incubation, the basal medium was recovered to determine the concentration of permeated gluten peptides by enzyme-linked immunosorbent assay (ELISA), as described below.

Gluten quantification. A commercially available quantitative immune-based ELISA kit, designed to detect the toxic fraction of gluten from food samples, was used according to the manufacturer's instructions (GlutenTox; Biomedal, Seville, Spain) (25). The analyses were performed in the fraction that reached the basal compartment after crossing the monolayer of Caco-2 cells subjected to inflammatory conditions and the gliadin samples digested in gastrointestinal conditions and in the presence or absence of selected *B. fragilis* strains.

Analysis of inflammatory markers. In the supernatants of Caco-2 cell cultures exposed to the dialysates from digests of gliadins inoculated with the different *B. fragilis* strains, TNF-α (eBioscience, San Diego, CA), and IL-1β (eBioscience) were determined by ELISA according to the manufacturer's instructions. The sensitivity for these methods is 4 pg/ml.

Statistical analyses. The Renyi diversity index was used to explore differences in *Bacteroides* species among the different child groups. This index provides three further diversity index values: species richness (S), the Shannon index (H'), and the Simpson index (1-D) (44).

The chi-square test was used to establish differences in the abundance of *Bacteroides* spp. and virulence genes. A P value of <0.05 was considered statistically significant. The Bonferroni adjustment test was applied to correct the significance for multiple comparisons among the three child groups studied (active and nonactive CD patients and controls), which has the advantage of reducing type I errors and the disadvantage of increasing type II errors.

For experimental studies with Caco-2 cell cultures, one-way analysis of variance (ANOVA) and the Fisher least significant difference (LSD) *post hoc* test were applied. Statistical significance was established at $P < 0.05$, using the SPSS software (v.15; SPSS, Inc., Chicago, IL).

RESULTS

Diversity of *Bacteroides* spp. in the fecal microbiota of CD patients. The species richness (S), Shannon (H'), and Simpson (1-D) indexes were very similar between active CD patients ($S = 12$, $H' = 1.95$, and 1-D = 0.82), nonactive CD patients ($S = 14$, $H' = 2.15$, and 1-D = 0.84) and controls ($S = 13$, $H' = 2.09$, and 1-D = 0.84), indicating a similar diversity distribution between the studied child groups. Renyi diversity curves showed that active CD patients had a lower diversity than controls, whereas the diversity curves from nonactive CD patients intersected with the diversity curves from both active CD patients and controls and therefore could not be compared (Fig. 1).

The total number of clones ($n = 274$) recovered from feces of healthy subjects and CD patients that were identified as *Bacteroides* spp. or *Parabacteroides* spp. is shown in Table 2. *B. fragilis* was more frequently isolated from CD patients with either active ($P = 0.007$) or nonactive ($P = 0.009$) disease than from healthy controls. This species represented up to 12% of the total clones from both groups of CD patients, which differs markedly from the 1% isolated from the controls. *Parabacteroides distasonis* was more frequently isolated from active CD patients than from nonactive CD patients and controls ($P < 0.001$). In contrast, *Bacteroides ovatus* was more frequently detected in controls than in CD patients ($P = 0.014$), irrespective of the phase of the disease (active or nonactive), and *Bacteroides finegoldii* was more frequently isolated from controls than from active CD patients ($P = 0.014$).

Pathogenicity markers and proteolytic activity of *B. fragilis* strains. A total of 23 *B. fragilis* isolates were identified from the three groups of children and, according to RAPD-PCR fingerprint analyses, these included nine different strains (A to I). The carriage of *bft* and *mpII* virulence genes was analyzed in all isolates, and the same pattern was detected for isolates belonging to the same strain as defined by RAPD-PCR. The virulence gene carriage in the different *B. fragilis* strains is summarized in Table 3. The *bft* gene was detected in 13 *B. fragilis* clones from CD patients, 6 of which (identified as strain E or G) were associated with active disease and seven (strain H or I) with nonactive disease. Considering the *bft*-positive clones, the *bft-2* isoform was significantly more prevalent (85%) than the isoforms *bft-1* (15%, $P = 0.007$)

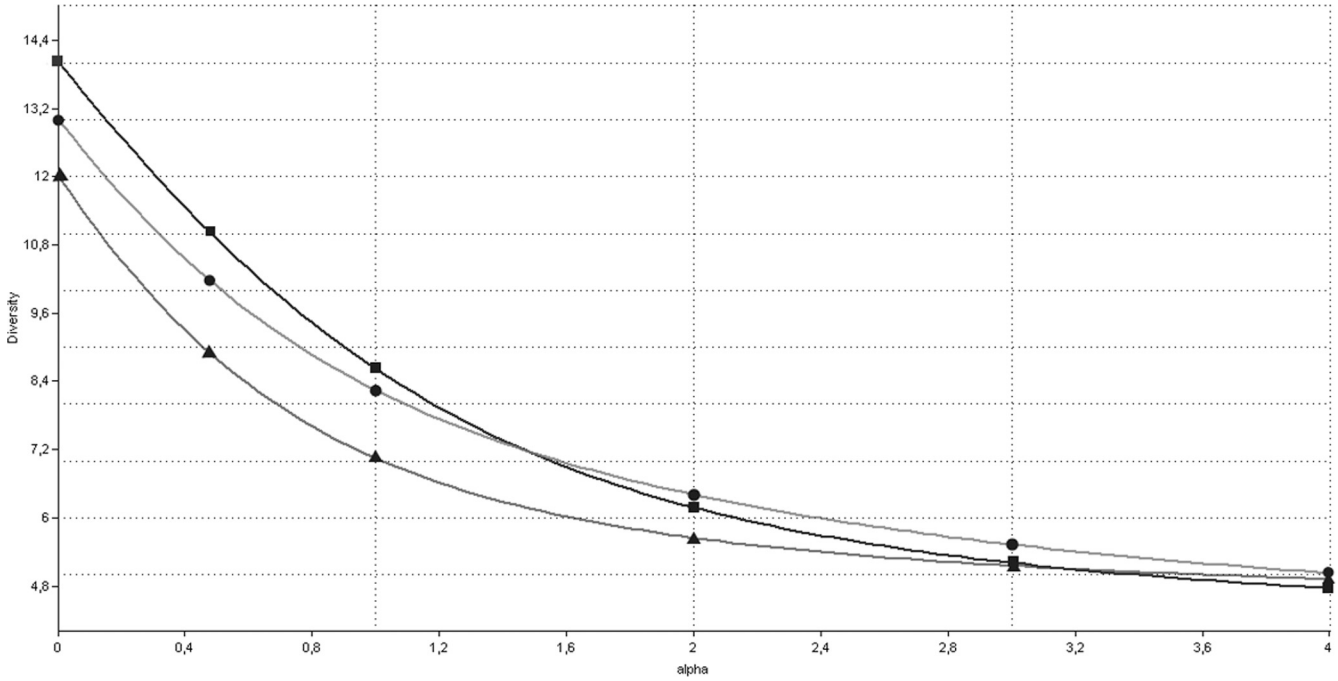

FIG 1 Renyi index curves of the *Bacteroides* spp. in active CD patients (▲), nonactive CD patients (■), and control children (●). The Renyi index estimates total richness for $\alpha = 0$, the Shannon index for $\alpha = 1$, and the Simpson index for $\alpha = 2$.

and *bft-3* (0%, $P < 0.001$). The *bft-2* isoform was detected in all of the *bft*-positive *B. fragilis* clones (strains E, G, and H) isolated from active and nonactive CD patients, whereas the *bft-1* isoform was only detected in two *bft*-positive clones (strain I) isolated from nonactive CD patients that also carried the *bft-2* isoform. The *mpII* gene was detected in three of the *B. fragilis* clones (strains B and D) isolated from active CD patients. Only one *B. fragilis* clone (strain A) was identified in the healthy controls, and it was *bft* and *mpII* negative.

In zymograms of gelatin, a common clear band of ~20 kDa was detected for both *bft*- or *mpII*-positive *B. fragilis* strains (B, D, E, G, H, and I) representing all isolated clones; the clearest bands corresponded to *B. fragilis* strains B and H. In contrast, hydrolysis bands were not detected in gelatin gels corresponding to the *B. fragilis* strains A, C, F, and J, which were *bft* and *mpII* negative (data not shown). In zymograms of gliadins, a proteolytic band of

TABLE 2 *Bacteroides* and *Parabacteroides* spp. isolated from fecal samples of active and nonactive CD patients and control children

Species	Species abundance (%)[a]		
	Active CD ($n = 97$)	Nonactive CD ($n = 82$)	Control ($n = 95$)
B. acidofaciens	0^{A}	$2\ (2.4)^{A}$	$2\ (2.1)^{A}$
B. caccae	$1\ (1.0)^{A}$	$4\ (4.9)^{A}$	$6\ (6.3)^{A}$
B. dorei	$4\ (4.1)^{A}$	$4\ (4.9)^{A}$	$11\ (11.6)^{A}$
B. finegoldii	0^{A}	$1\ (1.2)^{AB}$	$7\ (7.4)^{B}$
B. fragilis	$12\ (12.4)^{A}$	$10\ (12.2)^{A}$	$1\ (1.1)^{B}$
B. intestinalis	$1\ (1.0)^{A}$	$3\ (3.7)^{A}$	0^{A}
B. massiliensis	$2\ (2.1)^{A}$	0^{A}	0^{A}
B. ovatus	$5\ (5.2)^{A}$	$4\ (4.9)^{A}$	$16\ (16.8)^{B}$
B. stercoris	$1\ (1.0)^{A}$	$1\ (1.2)^{A}$	$2\ (2.1)^{A}$
B. thetaiotaomicron	$1\ (1.0)^{A}$	$2\ (2.4)^{A}$	$1\ (1.1)^{A}$
B. uniformis	$22\ (22.7)^{A}$	$23\ (28.0)^{A}$	$27\ (29.5)^{A}$
B. vulgatus	$20\ (20.6)^{A}$	$19\ (23.2)^{A}$	$13\ (13.7)^{A}$
B. xylanisolvens	0^{A}	$4\ (4.9)^{A}$	$2\ (2.1)^{A}$
P. distasonis	$24\ (24.7)^{A}$	$4\ (4.9)^{B}$	$6\ (6.3)^{B}$
P. merdae	$4\ (4.1)^{A}$	$1\ (1.2)^{A}$	$1\ (1.1)^{A}$

[a] That is, the percentage of clones belonging to one specific species related to the total number of clones isolated from each child group (active CD patients, non-active CD patients or controls). Superscript letters (A and B) indicate the statistical differences calculated by using the chi-square test (2 × 2), corrected for a multiple-comparison test (three child groups) by using the Bonferroni adjustment. Significant differences between groups were defined as $P < 0.017$.

TABLE 3 Characterization of virulence-associated genes (*bft* and *mpII*) in *B. fragilis* strains isolatedfrom active CD patients, nonactive CD patients, and control children

Source and *B. fragilis* strain (no. of clones)	Presence (+) or absence (−) of virulence genes	
	bft	*mpII*
Control children		
Strain A (1)	−	−
Active CD patients		
Strain B (2)	−	+
Strain C (2)	−	−
Strain D (1)	−	+
Strain E (2)	+	−
Strain F (1)	−	−
Strain G (4)	+	−
Nonactive CD patients		
Strain F (3)	−	−
Strain H (2)	+	−
Strain I (5)	+	−

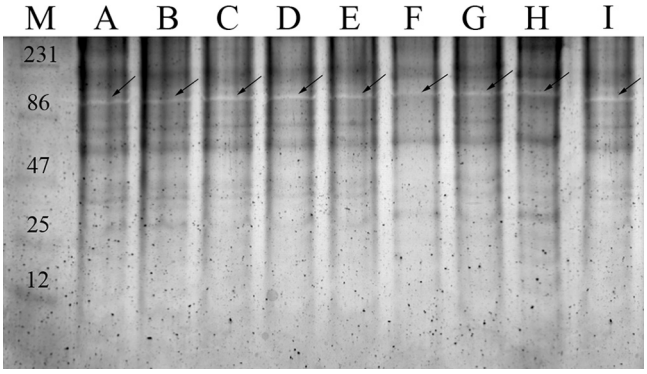

FIG 2 Gliadin zymograms of the *B. fragilis* strains (lanes A to I) without EDTA. The identified gliadin-degrading protease pattern is characterized by one clear band (arrow), present in all of the samples with an approximate molecular mass of 120 kDa. Lane M, molecular masses of the protein markers in indicated in kilodaltons.

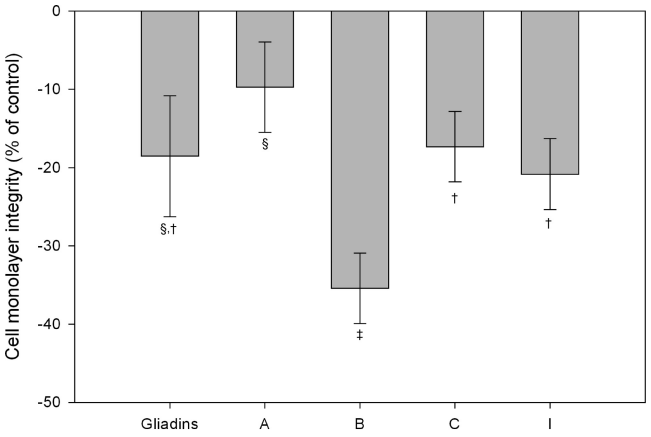

FIG 3 Effect of the dialyzed fraction of gliadins digested in the presence of several *B. fragilis* strains on sensitized (TNF-α) Caco-2 cells monolayer permeability to phenol red after 4 h of incubation. Values are expressed as means ± the standard deviations ($n = 4$). Different symbols (†, ‡, and §) indicate statistically significant differences ($P < 0.05$) among the treatments tested (gliadin and strains A, B, C, and I) by applying ANOVA and the LSD *post hoc* test. In addition, effects of all treatments were significantly different from those of controls (nonstimulated cells [data not shown]).

~120 kDa was detected in all of the *B. fragilis* strains tested (Fig. 2). These activities were partially or totally inhibited in the presence of EDTA (data not shown), indicating that metalloproteases were responsible for the gliadin hydrolysis in the gels. According to these results, *B. fragilis* strains A, B, C, and I, which represent strains of different origins and gene carriages, were selected for further studies on their possible differential pathogenic effects on intestinal epithelial cells and ability to hydrolyze gliadin peptides.

Effects of *B. fragilis* strains on monolayer integrity of Caco-2 cells. The possible direct adverse effects of the selected *B. fragilis* strains on cell monolayer integrity were evaluated by a phenol red diffusion assay, whereby diffusion takes place only across the tight junctions (Fig. 3). There was a significant increase ($P < 0.05$) in the basal content of phenol red in Caco-2 cell cultures incubated with *B. fragilis* strains B, C, and I compared to control cultures (data not shown), indicating alteration of the integrity of the cell monolayer. *B. fragilis* strain B (positive for the *mpII* gene and found in active CD patients) and, secondarily, *B. fragilis* strain I (positive for *bft*) caused the most marked impairment of cell monolayer integrity.

Effects of *B. fragilis* strains in the generation of gliadin-derived peptides during digestion. The amino acid sequences of gliadin-derived peptides generated during simulated gastrointestinal digestion, in the presence or absence of the selected *B. fragilis* strains, and that cross the dialysis membrane, are shown in Table 4. The total protein content of the dialysates from *in vitro* gliadin digestions, not inoculated with *B. fragilis* strains, was 1.05 ± 0.16 mg, representing up to 7% of the total protein loaded in the upper chamber of the *in vitro* system. However, inoculation of the selected *B. fragilis* strains during digestions increased the total dialyzable protein fraction to 2.27 ± 0.38 mg, which constitutes up to 13.3 to 18.7% of the total protein content in the upper chamber, indicating an increase in the degree of gliadin hydrolysis. Peptides generated during *in vitro* digestions showed high variability in their molecular masses, which ranged from 465.7 to 4,869.8 Da. In samples of gliadins digested in the absence of *B. fragilis* strains, peptides with amino acid sequences such as α/β-gliadin from amino acid positions 80 to 89 (α/β-Gld[80-89]) and α/β-Gld[80-100], which are inflammatory, and α/β-Gld[124-133], which has an amino acid sequence similar as those that interact with the chemokine receptor CXCR3, were identified (18, 20). In samples inoculated with the different *B. fragilis* strains different peptides with amino acid sequences of the main epitopes found in the immunodominant 33-mer peptide of α-Gld[56-88] were identified. For example, the peptide sequences for α/β-Gld[62-81], α/β-Gld[82-90], and α/β-Gld[77-85] were identified in gliadin samples digested in the presence of *B. fragilis* strain A; the sequences for α/β-Gld[84-96] and α/β-Gld[72-87] were identified in gliadin samples digested in the presence of *B. fragilis* strain I; the sequences for α/β-Gld[56-68], α/β-Gld[77-94], and α/β-Gld[50-86] were identified in gliadin samples digested in the presence of *B. fragilis* strain C; and the sequences for α/β-Gld[54-94] and α/β-Gld[82-90] were identified in gliadin samples digested in the presence of *B. fragilis* strain B. The molecular masses of the peptides identified could not be associated with *bft* or *mp II* gene carriage, but the shorter peptides were identified in samples inoculated with the strains *B. fragilis* strains A and I.

Inflammatory cytokine production by intestinal Caco-2 cells exposed to digested gliadins. TNF-α and IL-1β production by Caco-2 cell cultures exposed to gliadin digestions, inoculated or not with the different *B. fragilis* strains selected, are shown in Fig. 4. After gastrointestinal digestion, gliadin-derived peptides significantly induced ($P < 0.05$) TNF-α and IL-1β production in the presence or absence of all of the *B. fragilis* strains tested in comparison to the controls. *B. fragilis* strains B and C induced the highest TNF-α production, whose values were significantly ($P < 0.05$) higher than those induced by gliadins digested alone, indicating that these *B. fragilis* strains could increase the gliadin-mediated proinflammatory potential. *B. fragilis* strains C and I induced the highest production of IL-1β, but the increase was only significant in comparison to the control and not compared to gliadin digested without bacteria.

Effect of *B. fragilis* strains on the permeability of the Caco-2 cell monolayer to gliadins. To evaluate whether *B. fragilis* strains could increase the permeability of Caco-2 cells to gliadin peptides by their direct deleterious effects on intestinal Caco-2 cell monolayer integrity and by increasing the amount of dialyzable and

TABLE 4 Gliadin-derived peptides in dialyzed fractions from different gastrointestinal digestions of gliadins, inoculated or not with *B. fragilis* strains

Gliadin/*B. fragilis* strain	Peptide	Amino acid sequence	Observed *m/z*	Calculated *m/z*	Ion (*m/z*) selected for MS/MS (charge)
Gliadins only	α/β-Gld[90-93]	QPQP	465.7	468.3	464.7 (1)
	α/β-Gld[82-89]	PQPQLPYP	933.4	928.5	932.4 (1)
	α/β-Gld[217-224]	SQVSFQQPQ	1,033.4	1,033.5	1,032.4 (1)
	α/β-Gld[124-133]	QQQQQQILQQ	1,270.3	1,269.7	1,270.0 (1)
	α/β-Gld[75-87]	YLQLQPFPQPQLPYPQ	1,472.7	1,471.8	1,471.7 (1)
	α/β-Gld[75-92]	YLQLQPFPQPQLPYPQPQ	2,172.8	2,172.1	1,085.9 (2)
	α/β-Gld[80-100]	PFPQPQLPYPQPQPFRPQQPY	2,541.7	2,541.8	1,270.4 (2)
Gliadins plus strain:					
A	γ-Gld[24-46]	QPFSQQPQQIFPQPQQTPHQPQQ	2,371.5	2,370.2	2,371.5 (1)
	α/β-Gld[62-81]	YPQPQPFPSQQPYLQLQPF	2,402.0	2,400.2	2,402.0 (1)
	α/β-Gld[82-90]	PQPQLPYPQ	1,067.5	1,068.2	1,067.5 (1)
	α/β-Gld[239-248]	QNPQAQGSFQ	1,106.5	1,105.1	1,106.5 (1)
	α/β-Gld[77-85]	QLQPFPQPQ	1,085.0	1,083.2	1,085.0 (1)
	ω-Gld[258-267]	QQPQQPYPQQ	1,240.9	1,241.6	1,240.9 (1)
	α/β-Gld[97-105]	QQPYPQPQP	1,082.9	1,082.5	1,082.9 (1)
B	α/β-Gld[248-279]	FQPQQLPQFEAIRNLALQFLPAMCNVYIPPYC	3,708.6	3,706.8	1,854.3 (2)
	γ-Gld[108-126]	QQSFPQQQPSLIQQSLQQ	2,242.9	2,242.4	2,242.9 (1)
	α/β-Gld[54-94]	QQPFPPQQPYPQPQPFPSQQPYLQLQPFPQPQLPYPQPQPF	4,869.8	4,868.4	2,434.9 (2)
	α/β-Gld[254-272]	PQFEAIRNLALQTLPAMCN	2,131.0	2,130.1	1,065.5 (2)
	α/β-Gld[82-90]	PQPQLPYPQ	1,067.9	1,068.2	1,067.9 (1)
C	γ-Gld[82-95]	FPQTQQPQQPFPQS	1,658.7	1,658.8	1,659.7 (1)
	ω-Gld[376-391]	YPQQQPYGSSLTSIGG	1,683.1	1,683.8	1,684.1 (1)
	γ-Gld[174-190]	QQLQCAAIHSVVHSIIM	1,877.3	1,879.2	1,878.3 (1)
	α/β-Gld[42-62]	VPLVQQQQFPGQQQPFPPQQP	2,416.2	2,416.2	1,208.6 (2)
	α/β-Gld[56-68]	PFPPQQPYPQPQP	1,520.8	1,521.7	1,521.8 (1)
	α/β-Gld[77-94]	QLQPFPQPQLPYPQPQPF	2,149.3	2,150.1	2,150.3 (1)
	α/β-Gld[50-86]	FPGQQQPFPPQQPYPQPQPFPSQQPYLQLQPFPQPQL	4,343.3	4,343.2	1,448.1 (3)
I	α/β-Gld[207-231]	QQQQQQQQPLSQVSFQQPQQQYPSG	2,944.4	2,943.4	981.8 (3)
	ω-Gld[338-355]	PQQPFQQPQQQLSQQPEQ	2,165.6	2,165.3	2,166.6 (1)
	α/β-Gld[97-113]	QQPYPQPQPQYSQPQQP	2,041.3	2,040.2	2,042.3 (1)
	α/β-Gld[229-246]	PSGQGFFQPSQQNPQAQG	1,902.9	1,903.9	1,903.9 (1)
	α/β-Gld[148-167]	QQHNIAQGRSQVLQQSTYQL	2,329.2	2,328.5	2,330.2 (1)
	ω-Gld[355-371]	QTISQQPQQPFPQQPHQ	2,018.2	2,018.2	2,019.2 (1)
	α/β-Gld[84-96]	PQLPYPQPQPFRP	1,565.3	1,565.8	1,566.3 (1)
	α/β-Gld[72-87]	QQPYLQLQPFPQPQLP	1,924.8	1,923.2	1,925.8 (1)

soluble fraction of gliadin through proteolytic degradation, Caco-2 cells were exposed simultaneously to the bacterial cell suspensions and the dialyzable fraction of gliadins digested in the presence of each *B. fragilis* strain. The toxic fraction of gluten initially loaded in the apical part of the two-chamber system was quantified by ELISA and also in the basal compartment after being incubated with the bacterial strains located in the apical compartment of the two-chamber system. The initial toxic fraction of gluten was significantly ($P < 0.05$) lower in the samples of gliadins digested alone (104.9 ± 4.4 μg/g) than in samples of gliadins digested in the presence of *B. fragilis* strains A (124.1 ± 1.1 μg/g), B (126.4 ± 1.9 μg/g), C (124.8 ± 3.3 μg/g), and I (118.4 ± 1.3 μg/g). The toxic fractions of gluten detected in the basolateral compartment of Caco-2 cultures exposed to these gliadin digestions, together with the cell suspensions of *B. fragilis* strains, were higher ($P < 0.05$) than in cell cultures exposed to gliadins digested without bacteria (Fig. 5). *B. fragilis* strain B caused the highest increase in gluten permeability in Caco-2 cells, probably due to its having the most deleterious effect on monolayer integrity related to the presence of the *mpII* gene and its ability to generate peptides that activated a stronger inflammatory response. This strain also generated the most immunogenic 33-mer (α/β-Gld[54-94], Table 4) identified in α-gliadin.

DISCUSSION

This study reports on the composition and potential virulence features of intestinal *Bacteroides* spp. in CD patients in comparison to healthy controls. *Bacteroides* spp. are commensal inhabitants of the human gastrointestinal tract, but have also been associated with chronic inflammatory bowel disorders (IBDs), such as ulcerative colitis and Crohn's disease, (2, 41, 43) and with CD (8, 9, 26). In IBD patients, bacteroides and enterobacteria have been considered responsible for >60% of the biofilm mass in the mucosa (41). Our study shows that active CD patients had a higher abundance of *B. fragilis* and a lower abundance of *B. ovatus* than controls, and these differ-

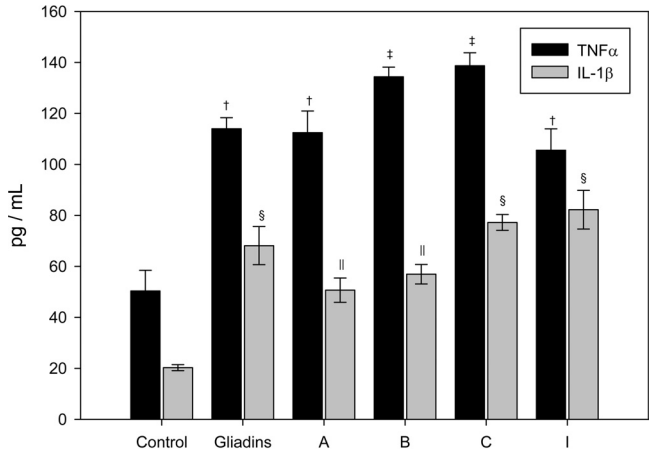

FIG 4 TNF-α and IL-1β production by Caco-2 cell cultures exposed to the dialyzed fraction of gliadins digested in the presence of several *B. fragilis* strains. The results are expressed as means ± the standard deviations ($n = 5$). Different symbols indicate statistically significant differences ($P < 0.05$) for TNF-α († and ‡) or for IL-1β (§ and ||) by applying ANOVA and the LSD *post hoc* test.

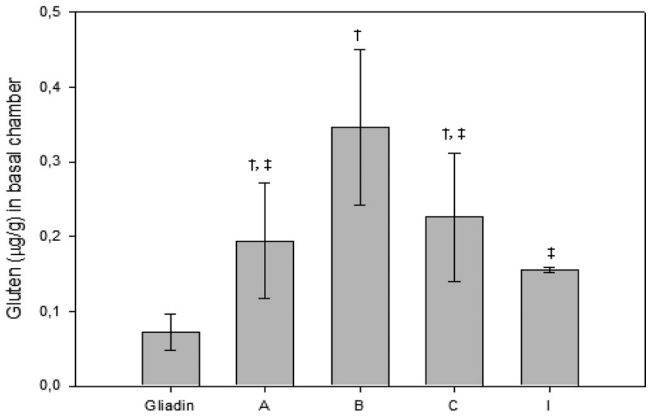

FIG 5 Gluten content quantified in the basal chamber of Caco-2 cell cultures exposed to gliadins digested in the presence of cell suspensions of the different *B. fragilis* strains. Values are expressed as means ± the standard deviations ($n = 4$). Different symbols († and ‡) indicate statistically significant differences ($P < 0.05$) among the different strains tested by applying ANOVA and the LSD *post hoc* test. In addition, effects of all strains (A, B, C, and I) were significantly different from those detected in cells exposed to gliadin alone.

ences were not restored after long-term adherence to a gluten-free diet, suggesting this could play a primary role in the disease. Nevertheless, differences in the abundance of *P. distasonis* and *B. finegoldii* between active CD patients and controls were restored after adherence to the gluten-free diet. Although differences between fecal and duodenal mucosal bacteria may exist, our previous studies reported that *B. fragilis* numbers of feces and biopsy specimens from CD patients correlated, and their differences in comparison with healthy controls were similar (8), justifying the present study conducted with bacteroides from feces. In accordance with our results, *B. fragilis* is one of the least common species inhabiting the intestinal tract of healthy subjects, and yet it is most frequently isolated from clinical specimens and is the most virulent species (46). In contrast, *B. vulgatus* has been generally considered one of the most common intestinal *Bacteroides* species of healthy subjects (46), although in our study no such association was found. Nevertheless, other human studies have also reported associations between increased abundance of *B. vulgatus* and/or *B. ovatus* and chronic IBDs (10, 22), which partly contrast with our findings in CD patients.

It is known that the pathogenic potential of *Bacteroides* spp. depends on the presence of different virulence factors (e.g., agglutinins, polysaccharide capsules, or lipopolysaccharides) and a variety of proteolytic and hydrolytic enzymes (29, 46). Moreover, the comparative analyses of the whole genomes of several *B. fragilis* strains are revealing even larger genomic differences among strains (e.g., polysaccharide biosynthesis), which could determine their different virulence as opportunistic pathogens and ability to evade the immune defense mechanisms (27, 28). This evidence stresses the need to characterize the isolates at strain level and determine their specific virulence features to understand their potential pathogenicity in a specific ecosystem. In our study, the presence of genes encoding for metalloproteases was evaluated. *B. fragilis* clones with genes encoding for metalloproteases were frequently isolated from CD patients, which suggests a role of at least this species and probably these genes in disease pathogenesis according to our preliminary *in vitro* studies. Enterotoxigenic *B. fragilis* (ETBF) strains have been related to the inflammatory process in humans (2, 30) or animal models of IBD (30, 32). The enterotoxin produced by ETBF strains is encoded by the *bft* gene, which can be in three different isoforms; *bft-1*, *bft-2*, and *bft-3* (37). In the present study, *bft-2* was the most common isoform detected in the *B. fragilis* clones isolated from CD patients, which also seemed to be the most frequently present in clones that colonize the guts of children in comparison to those that colonize adult guts (36). The *B. fragilis* strains tested exhibited a genetic pattern where only either the *bft* gene or the *mpII* gene was present. In accordance with the size of the protease bands detected in our zymograms (Fig. 1), the *bft* gene encodes a zinc-dependent *B. fragilis* toxin (BFT), which is translated as a prepro-protein (44.4 kDa) further processed to a biologically active toxin of 168 amino acid residues, with a molecular mass of 20.7 kDa (13). In addition, the *mpII* gene also encodes a metalloprotease (20 kDa) predicted to be a zinc-dependent protein with 56% similarity to BFT protein (12).

The BFT is a soluble virulence factor secreted to the extracellular medium (13) that increases permeability of the intestinal epithelium by cleavage of the trans-membrane adhesion protein E-cadherin (47). A reduced expression of E-cadherin has been reported in CD patients, (1), although its relation to the microbiota has not yet been directly established. Our study suggests that the carriage of metalloprotease virulence genes by *B. fragilis* strains is associated with the ability of these strains to increase the permeability of the intestinal epithelium *in vitro* and that tight junctions are one of the early sites injured in Caco-2 cell cultures. This property was associated with both *bft* and *mpII* gene carriage, but the most remarkable effects were detected in the strain carrying the *mpII* gene. It has been found that both *bft-1/bft-2* isoforms and *mpII* genes are clustered in pathogenicity islands, (12) and suggested that MPII is synthesized as a precursor protein similar to BFT (13). The highly conserved sequence of MPII found in different *B. fragilis* strains has led to hypotheses of important roles for this protein in ETBF strains and/or in ETBF-induced disease, although, to the best of our knowledge, no biological activity has yet

been identified for this protein. *B. fragilis* strains not endowed with these metalloprotease genes and gelatinase activity also reduced the integrity of the Caco-2 cell monolayer to some extent, suggesting that other factors may also be responsible for this effect.

All of the *B. fragilis* strains studied exhibited gliadin-hydrolyzing activity. In a previous study, gliadin-hydrolyzing activity from microbial origin was found in biopsy specimens of CD patients, in contrast to controls, suggesting a pathogenic role for this activity, although this has yet to be confirmed (3). For this reason, we also evaluated whether *B. fragilis* could modify the peptide generated from gliadins and their potential immunotoxicity. Our study shows that the *B. fragilis* strains studied hydrolyzed gliadins, producing several peptides with the immunogenetic amino acid sequences of the main epitopes of the immunodominant 33-mer of α-Gld[56-88], while the partial digestion of gliadins by the gastrointestinal proteolytic enzymes used in the *in vitro* system did not produce these particular toxic sequences (19). In order to understand the possible pathological consequences of the hydrolytic activities of *B. fragilis* strains in this disorder, the inflammatory effects of the peptides generated were evaluated, and we demonstrated that they preserve or even increase their ability to induce inflammatory cytokine production (TNF-α). These increases in TNF-α production by epithelial cells could have adverse consequences on the pathogenesis of CD because this cytokine, in conjunction with IL-1β, increases paracellular permeability, facilitating the translocation of immunogenic peptides derived from gliadin to the lamina propria (39), and also mediates the infiltration of lymphocytes in the intestinal epithelium, thereby promoting tissue inflammation (16).

Although all *B. fragilis* strains were shown to have gliadin-hydrolyzing activity, the effects of different *B. fragilis* strains and of the peptides generated were slightly different, probably due to different levels of expression of the possible enzymes responsible or slightly different specificities (6). In addition, the gliadin-hydrolyzing activity of all *B. fragilis* strains increased the degree of proteolytic degradation and lowered the molecular masses of the peptides generated, increasing the protein content in the bio-accessible fraction that could facilitate interactions with the apical chemokine CXCR3 receptor of enterocytes, triggering inflammatory events (18, 20). In addition, the permeation of immunogenic peptides generated by *B. fragilis* from α-gliadins across intestinal epithelia was favored, which could promote the interaction of the peptides with the tissue transglutaminase and with antigen-presenting cells that ultimately activate the T cells responsible for the full expression of the disease (34).

Conclusions. We have demonstrated here that the species *B. fragilis* is more abundant in the intestinal microbiota of CD patients, whereas *B. ovatus* is less abundant in comparison to healthy controls. These differences were also detected in CD patients after adherence to a gluten-free diet, suggesting that these alterations are not secondary to the underlying disease. We also demonstrated that *B. fragilis* clones carrying the *bft* and *mpII* metalloprotease genes and activity are abundant in CD patients and cause alterations in epithelial permeability. *B. fragilis* clones are also endowed with additional metalloproteases with gliadin specificity that generate immunotoxic peptides during *in vitro* intestinal digestion of gliadin. The generated peptides preserve or even increase their inflammatory properties on intestinal cells and more easily permeate the intestinal epithelial layer, which could favor their interaction with professional immunocompetent cells in the submucosa, although the magnitude of the effects are strain dependent. All in all, our findings indicate that the increased abundance of *B. fragilis* strains with metalloprotease activities in CD patients could play a pathogenic role, although evidence from *in vivo* studies is needed to confirm such a hypothesis.

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